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13. ABSTRACT (Maximum 200 Words)

The important role of polyamines in regulation of cell growth has led to the development of a number of polyamine analogues that can intervene in natural polyamine metabolism and inhibit the growth of tumor cells. This proposal was designed to elucidate the molecular mechanisms and the therapeutic efficacy of new generation of polyamine analogues in treatment of human breast cancer. In the first year of this award, we have performed the preliminary study of the *in vitro* and *in vivo* anti-tumor efficacy of novel polyamine analogues in breast cancer cells (*Clin. Cancer. Res.*, 9: 2769, 2003) and continued on the investigation of the molecular mechanisms responsible for the growth inhibition and apoptosis induced by polyamine analogues. Our studies indicate that polyamine analogue-inducible AP-1 plays a pro-survival role in polyamine analogue treated breast cancer cell. (*Mol. Cancer Res.*, 2: 81, 2004). Our studies also showed that activation of the p53 is important for the induction of polyamine analogue-induced growth inhibition and apoptosis, whereas JNK/Jun signaling pathway may negatively regulate p53 (unpublished). These data suggest that p53 and JNK/Jun pathways may be the useful molecular targets for improving the therapeutic efficacy of polyamine analogues in human breast cancer.

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INTRODUCTION

The natural polyamines (putrescine, spermidine and spermine) have been shown to be essential for cell growth. The important role of polyamines in regulation of cell growth has led to the development of a number of polyamine analogues that can intervene in natural polyamine metabolism and inhibit the growth of tumor cells. As a consequence, work has focused on the therapeutic development of inhibitors of the polyamine analogue metabolism pathway. Recently a new generation of polyamine analogues designated as oligoamines have been developed by creating longer chains than natural polyamine molecules. The purpose of this project is to elucidate the molecular mechanisms and therapeutic efficacy of a novel generation of polyamine analogues in the treatment of human breast cancer. This will be accomplished through three related technical objectives. They include: 1) to investigate *in vitro* molecular mechanisms responsible for the growth inhibition and apoptosis induced by novel polyamine analogues in breast cancer cells, 2) to expand the *in vitro* studies to an *in vivo* animal model of human breast cancer, 3) to study the therapeutic efficacy of combinations of novel promising polyamine analogues with clinically active antineoplastic agents.

BODY

Technical Objective 1: To investigate *in vitro* molecular mechanisms responsible for the growth inhibition and apoptosis induced by novel polyamine analogues in breast cancer cells. (month 1-16)

The study length of this specific objective was proposed for 16 months. When this proposal was funded in June 2003, we have already performed the preliminary analysis of the *in vitro* and *in vivo* anti-tumor activities of several novel polyamine analogues in human breast cancer. The results were published in the July/2004 issue of Clinical Cancer Research (*Clin. Cancer. Res.*, 9: 2769, 2003). Briefly, in that publication, we demonstrated that a novel polyamine analogue, SL-11144, has effective antineoplastic action against human breast cancer cells *in vitro* and *in vivo*, and multiple apoptotic mechanisms are associated with its cytotoxic effect in specific human breast cancer cell lines.

Based on our preliminary findings, we continued to investigate the molecular mechanisms of oligoamine induced growth inhibition and apoptosis. By use of a novel Jun NH_2-terminal Kinase (JNK) inhibitor, SP600125, we found that inhibition of c-Jun activity sensitized tumor cells to oligoamine-induced cell death in human breast cancer MDA-MB-435 cells, suggesting that JNK/Jun pathway may protect cells from oligoamine induced cell death. To directly test this hypothesis, cells were stably transfected with the dominant-negative mutant c-Jun (TAM67), which lacks the NH_2-terminal transactivation domain. Cells overexpressing TAM67 exhibit normal growth kinetics, but demonstrate a significantly increased sensitivity to oligoamine cytotoxicity and attenuated colony formation after oligoamine treatment. Furthermore, oligoamine treatment leads to more profound Caspase-3 activation and PARP cleavage in TAM67 transfectants, suggesting that c-Jun acts as an anti-apoptosis factor in MDA-MB-435 cells in response to oligoamine treatment. These findings indicate that

oligoamine-inducible AP-1 plays a pro-survival role in oligoamine treated MDA-MB-435 cells, and JNK/AP-1 might be a potential target for enhancing the therapeutic efficacy of polyamine analogues in human breast cancer. This work has been published in the February/2004 issue of Molecular Cancer Research (*Mol. Cancer Res.*, 2: 81, 2004).

In our latest studies in this specific aim, we demonstrated that p53/p21 activation is a common response in human breast cancer MCF-7 cells upon the treatment with a variety of structurally different polyamine analogues. Stable transfection of small interfering RNA (siRNA) targeting p53 blocked the expression of p21 induced by the polyamine analogues and significantly inhibited polyamine analogue-induced growth inhibition and apoptosis. Interestingly, the effects of analogue exposure on cyclins and cyclin dependent kinases varied with the specific agent used. The use of JNK inhibitor SP600125 or overexpression of dominant-negative mutant c-Jun (TAM67) sensitized MCF-7 cells to polyamine analogue-induced cell death and promoted the activation of p53/p21. Neither p38 inhibitor nor ERK inhibitor affected polyamine analogue-induced p53 activation and growth inhibition. These results suggest that activation of p53 is important for the induction of polyamine analogue-induced growth inhibition and apoptosis, whereas the JNK/Jun signaling pathway may negatively regulate p53 and play a protective role in MCF-7 cells in response to polyamine analogue treatment. Further examination of the impact of p53/p21 and JNK/Jun activation on polyamine analogue cytotoxicity is under investigation.

Technical Objective 2: To expand the *in vitro* studies to an *in vivo* animal model of human breast cancer (months17-26).

These studies will begin in the next year.

Technical Objective 3: To study the therapeutic efficacy of combinations of novel polyamine analogues with clinically active antineoplastic agents (months 27-36).

Implementation of this technical objective was not planned until year 3 as results from Technical Objective 2 are needed to guide the design of these studies.

KEY RESEARCH ACCOMPLISHMENTS

1. Identified the promising novel polyamine analogues, such as SL-11144, which exhibit *in vitro* and *in vivo* anti-tumor activities in human breast cancer cells.
2. Revealed the protective and anti-apoptotic roles of JNK/Jun signaling pathway in polyamine analogue induced growth inhibition and apoptosis.
3. Demonstrated that activation of p53/p21 is a common response in p53 wild type human breast cancer cells in response to the treatment of polyamine analogues with different chemical structures.

REPORTABLE OUTCOMES

1. Paper "Huang, Y., Hager, E.R., Phillips, D.L., Dunn, V.R., Hacker, A., Frydman, B., Kink J.A., Valasinas, A.L., Reddy, V.K., Marton, L.J., Casero, R.A., Davidson, N.E. A Novel Polyamine Analog Inhibits Growth and Induces

Apoptosis in Human Breast Cancer Cells. Clin. Cancer. Res., 9: 2769-2777, 2003."

2. Paper "Huang, Y., Keen, J., Hager, E.R., Smith, R., Frydman, B., Valasinas, A.L., Reddy, V.K., Marton, L.J., Casero, R.A., Davidson, N.E. Regulation of Polyamine Analogue Cytotoxicity by c-Jun in Human Cancer MDA-MB-435 Cells. Mol. Cancer Res., 2: 81-88, 2004."
3. Abstract "Yi Huang, Allison Pledgie, Ethel Rubin, Benjamin Frydman, Aldonia L. Valasinas, Venodhar K. Reddy, Laurence J. Marton, Patrick M. Woster, Robert A. Casero, Jr., Nancy E. Davidson. Distinct roles of p53/p21$^{WAF1/CIP1}$ and JNK/Jun activation in the mediation of polyamine analogue induced growth inhibition and cell death in human breast cancer MCF-7 cells." Accepted by 12th NIH SPORE Workshop (Poster discussion) in Baltimore, July 2004 and 2004 Gordon Research Conference on Molecular Therapeutics of Cancer (Poster) in New London, New Hampshire, July 2004.

CONCLUSIONS

A series of studies have been carried out to evaluate the *in vitro* molecular mechanisms responsible for the growth inhibition and apoptosis induced by novel polyamine analogues in breast cancer cells. Our initial investigations revealed the protective and anti-apoptotic roles of JNK/Jun signaling pathway in polyamine analogue induced growth inhibition and apoptosis. We also demonstrated that activation of p53/p21 is a common response in p53 wild type human breast cancer cells in response to the treatment of polyamine analogues with different chemical structures. Examination of the molecular mechanisms of polyamine analogues in human breast cancer cells will be continued and the investigation for objectives 2 & 3 will begin as scheduled.

REFERENCES

1. **Huang, Y.**, Hager, E.R., Phillips, D.L., Dunn, V.R., Hacker, A., Frydman, B., Kink J.A., Valasinas, A.L., Reddy, V.K., Marton, L.J., Casero, R.A., Davidson, N.E. A Novel Polyamine Analog Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells. Clin. Cancer. Res., 9: 2769-2777, 2003.
2. **Huang, Y.**, Keen, J., Hager, E.R., Smith, R., Frydman, B., Valasinas, A.L., Reddy, V.K., Marton, L.J., Casero, R.A., Davidson, N.E. Regulation of Polyamine Analogue Cytotoxicity by c-Jun in Human Cancer MDA-MB-435 Cells. Mol. Cancer Res., 2: 81-88, 2004.

APPENDICES

1. Copy of paper published in Clin. Cancer. Res., 9: 2769-2777, 2003.
2. Copy of paper published in Mol. Cancer Res., 2: 81-88, 2004.
3. Abstract accepted by 12th NIH SPORE Workshop (Poster discussion) and 2004 Gordon Research Conference on Molecular Therapeutics of Cancer

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A Novel Polyamine Analog Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells[1]

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ABSTRACT

Polyamine analogs have demonstrated considerable activity against many important solid tumor models including breast cancer. However, the precise mechanisms of antitumor activities of polyamine analogs are not entirely understood. The cytotoxicity of a newly developed polyamine analog compound, SL11144, against human breast cancer was assessed. Treatment of human breast cancer cell lines in culture with SL11144 decreased cell proliferation and induced programmed cell death in a time- and dose-dependent manner. SL11144 also profoundly inhibited the growth of MDA-MB-231 xenografts in host nude mice without overt toxic effects. Treatment of MDA-MB-435 cells with SL11144 led to the release of cytochrome *c* from mitochondria into cytosol, activation of caspase-3, and poly(ADP-ribose) polymerase cleavage. SL11144 decreased Bcl-2 and increased Bax protein levels in MDA-MB-231 cells. Furthermore, activator protein 1 transcriptional factor family member c-Jun was up-regulated by SL11144 in MDA-MB-435 and MDA-MB-231 cells, but not in MCF7 cells. In addition, significant inhibition of ornithine decarboxylase activity and a decrease in polyamine pools were demonstrated. These results demonstrate that the novel polyamine analog SL11144 has effective antineoplastic action against human breast cancer cells *in vitro* and *in vivo* and that multiple apoptotic mechanisms are associated with its cytotoxic effect in specific human breast cancer cell lines.

INTRODUCTION

The natural polyamines (Put,[3] Spd, and Spm) have been shown to be essential for cell growth. The critical role of polyamines in regulation of cell growth has led to the development of a number of inhibitors of key enzymes in the polyamine biosynthetic pathway as a therapeutic strategy (1–3). It has also been demonstrated that synthetic polyamine analogs can down-regulate polyamine biosynthesis by feedback mechanisms but are unable to act as substitutes for natural polyamines to promote cell growth. This approach has become an important means for the study of the physiological roles of natural polyamines and a potent application for creation of new antineoplastic agents (4–6). Indeed, several synthetic polyamine analogs have been reported to inhibit cell proliferation and induce PCD in a variety of tumor cell lines (7–11).

Apoptotic cell death is characterized by chromatin condensation, cytoplasmic blebbing, and internucleosomal DNA fragmentation and occurs in a variety of cellular systems in response to many different stimuli (12). We have demonstrated previously (9) that some polyamine analogs can induce PCD in hormone-responsive or -unresponsive human breast cancer cells. A highly regulated metabolic pathway finely controls intracellular polyamine concentrations. The rate-limiting enzymes ODC and S-Adenosylmethionine decarboxylase regulate biosynthesis, whereas catabolism is regulated by SSAT and human polyamine oxidase h1/spermine oxidase (13). Cell type-specific superinduction of SSAT and the subsequent depletion of natural polyamine pools have been reported in polyamine analog-induced growth inhibition and apoptosis in some tumor cell lines (9, 14). However, in other cell lines, polyamine analogs that do not highly induce SSAT can still inhibit tumor cell growth and produce apoptosis (15, 16). These divergent results suggest that polyamine analog-induced cell death may result from several agent-dependent mechanisms.

SL11144, a leading agent of a new generation of polyamine analogs designated as oligoamines, has shown significant activity against proliferating cells (17). In this study, we have evaluated the antineoplastic efficacy of SL11144 in human breast cancer cells *in vitro* and *in vivo*. The data presented in this study

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[2] To whom requests for reprints should be addressed, at CRB 409, Breast Cancer Research Program, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231. Phone: (410) 955-8489; Fax: (410) 614-4073; E-mail: davidna@jhmi.edu.

[3] The abbreviations used are: Put, putrescine; Spd, spermidine; Spm, spermine; PCD, programmed cell death; ODC, ornithine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase; BENSpm, N^1,N^{11}-bis(ethyl)norspermine; CPENSpm, N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8,-diazaundecane; CHENSpm, N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8,-diazaundecane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; AP-1, activator protein 1; PVDF, polyvinylidene difluoride; FasL, Fas ligand.

suggest that SL11144 significantly inhibits growth and induces PCD in human breast cancer cells.

MATERIALS AND METHODS

Compound, Cell Lines, and Culture Condition. The polyamine analog SL11144 (Fig. 1) was provided by SLIL Biomedical Corp. (Madison, WI). Polyamine analog compounds BENSpm, CPENSpm, and CHENSpm were synthesized as described previously (18). Concentrated stock solutions (10 mM in double-distilled H_2O) of polyamine analogs were diluted with medium to the indicated concentrations. Human breast cancer MDA-MB-231 and MCF7 cells were maintained in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. MDA-MB-435 cells were maintained in improved modification of eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

MTT Survival Assays. MTT assays were performed using a method described previously (19). Briefly, 2000–5000 cells were plated in 96-well dishes and treated with the various concentrations of SL11144 for different lengths of time. At the end of each time point, 100 μl of a 1 mg/ml MTT solution (Sigma Chemical Co., St. Louis, MO), diluted in serum-free culture media, were added to each well. The plates were incubated at 37°C in 5% CO_2 atmosphere for 4 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. At the end of the 4-h incubation, the MTT solution was removed, and 200 μl of 1:1 (v/v) solution of DMSO:ethanol were added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at $A_{540\ nm}$. All of the experiments were plated in quadruplicate, and the results of assays were presented as means ± SD.

Analysis of Intracellular Polyamine Pools, SSAT Activity, and ODC Activity. The intracellular polyamine content of treated and untreated cells was determined by precolumn dansylation and reversed phase high-performance liquid chromatography (20). SSAT and ODC activities were measured using cellular extracts as described previously (21, 22). Protein concentrations were determined according to the method of Bradford (23).

Hoechst Staining of Nuclear Chromatin. SL11144-treated cells were fixed with 4% formaldehyde in PBS at 37°C for 10 min and permeabilized with a 19:1 mixture of ethanol/acetic acid at –20°C for 15 min. Fixed cells were stained with 1 μg/ml Hoechst 33258 (Sigma Chemical Co.) in PBS at room temperature for 20 min. Hoechst staining of the cells was analyzed by fluorescence microscopy.

Determination of Internucleosomal DNA Cleavage. After tumor cells were treated with increasing concentrations of SL11144 for increasing times, cells were harvested, counted, and washed with PBS at 4°C. Cells were then suspended in lysis buffer (5 mM Tris-HCl, 20 mM EDTA, and 0.5% Triton X-100) and incubated for 20 min on ice. After incubation, samples were centrifuged at 14,000 × *g* for 20 min, and the supernatant was transferred to a reaction tube followed by phenol/chloroform/isoamyl (25:24:1) extraction. Two volumes of 100% ethanol were added to supernatant, followed by 5-min centrifugation at 14,000 × *g*. The pellet was resuspended in 0.1× SSC buffer and incubated with RNase for at least 30 min at 37°C. Then 50 μl of 5 M NaCl was added, followed by phenol/chloroform/isoamyl (25:24:1) extraction. After ethanol precipitation and centrifugation, the pellet was washed with 70% ethanol and dried. DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 μg/ml ethidium bromide and visualized under UV illumination. This method isolates only DNA ladder fragments without genomic DNA.

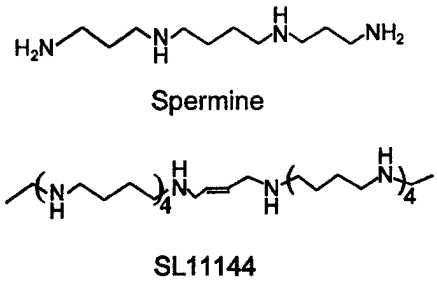

Fig. 1 Structures of Spm and polyamine analog SL11144.

Animal Studies. Female 4–6-week-old BALB c *nu/nu* athymic nude mice (Harlan Bioproducts for Science Inc., Indianapolis, IN) weighing between 16 and 18 g received injection in the right flank with 3.75×10^6 MDA-MB-231 cells. Cells were allowed to grow for 10 days to an average volume of 50–100 mm^3. Animals were then randomly assigned (eight mice for control group and seven mice for treatment groups) to receive vehicle control or SL11144 (2.5, 5, or 10 mg/kg) via i.p. injections twice weekly for 5 weeks. Tumor volumes were regularly assessed twice weekly by measuring 0.5 × length (mm) × width (mm) × width (mm). Mice were also weighed twice weekly.

Nuclear and Cytoplasmic Protein Extraction. The extractions of nuclear and cytoplasmic protein were performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL). MDA-MB-435 cells treated with 10 μM SL11144 for different times were harvested by trypsinization and washed with PBS. Two hundred μl of ice-cold Cytoplasmic Extraction Reagent I with protease inhibitors (0.5 mg/ml benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.2 M phenylmethylsulfonyl fluoride) were added to the cell pellets. After a 10-min incubation on ice, 11 μl of ice-cold Cytoplasmic Extraction Reagent II without protease inhibitors were added, followed by a 5-min centrifugation at 14,000 × *g*. The supernatant containing the cytoplasmic extract was retained, and the insoluble pellet was resuspended in 100 μl of Nuclear Extraction Reagent and incubated on ice for 40 min. After a 10-min centrifugation, the supernatant that contained the nuclear extract was saved. Both cytoplasmic and nuclear extracts were analyzed by Western blot using anti-c-Jun and anti-c-Fos antibodies as described below.

Detection of Cytochrome *c* Release. To avoid artifacts due to mechanical breakage of the outer mitochondrial membrane, selective plasma membrane permeabilization with digitonin was used to examine the release of cytochrome *c* from mitochondria into cytosol (24). Briefly, cells treated with dif-

ferent concentrations of SL11144 for the desired exposure time were harvested by trypsinization, washed with PBS, and subsequently incubated in 100 μl of permeabilization buffer [210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM succinate, 0.2 mM EGTA, and 100 μg/ml digitonin (pH 7.2)] for 5 min. After centrifugation for 10 min at 14,000 × *g*, the supernatant with protein content was saved, and protein concentrations were determined using the Pierce Micro Protein Assay Kit. Equal amounts of protein were fractionated using 12% SDS-PAGE and analyzed by Western blot as described below.

Western Blotting. Cells treated with different concentrations of SL11144 for the desired exposure times were harvested by trypsinization and washed with PBS. Cellular protein was isolated using the protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Protein concentrations were determined using the Pierce Micro Protein Assay Kit. Equal amounts of proteins (50 μg/lane) were fractionated using 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies against caspase-3, PARP, Bcl-2, Bax, caspase-8, caspase-9, cytochrome *c*, c-jun, c-fos, or FasL (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat antimouse or antirabbit secondary antibody (1:3000; DAKO Corp., Carpinteria, CA) followed by enhanced chemiluminescence staining using the enhanced chemiluminescence system (Amersham Biosciences). Actin was used to normalize for protein loading.

RESULTS

Inhibition of Growth by SL11144. The sensitivity of three human breast cancer cell lines (MDA-MB-231, MDA-MB-435, and MCF7 cells) to the newly synthesized polyamine analog SL11144 (Fig. 1) was assessed by using a MTT cellular survival assay. These cells were chosen because they represent hormone-responsive (MCF7) and -unresponsive (MDA-MB-231 and MDA-MB-435) human breast cancer cell lines. All three cell lines exhibited time- and concentration-dependent growth inhibition by SL11144 (Fig. 2, *A*–*C*). The IC_{50} values for MDA-MB-231 and MCF7 cells are about 1–5 μM for a 72-h treatment and 0.5–0.75 μM for a 96-h treatment. MDA-MB-435 cells were more sensitive to SL11144 with IC_{50} values of 2.5 μM for 24-h drug exposure and 0.25–0.5 μM for 48-h drug exposure. In addition, the cytotoxicity of SL11144 against MDA-MB-231 cells was compared with the identified polyamine analogs BENSpm, CPENSpm, and CHENSpm. MTT studies demonstrated that SL11144 had a lower IC_{50} at 96-h treatment in MDA-MB-231 cells than BENSpm (1–2.5 μM), CPENSpm (1–2.5 μM), or CHENSpm [~2.5 μM (data not shown)]. These data indicate that SL11144 is a more potent inhibitor of human breast tumor cell proliferation than previously synthesized polyamine analogs.

Regulation of Intracellular Polyamine Pools and Metabolic Enzymes by SL11144. To address whether the observed growth-inhibitory effects of SL11144 in human breast cancer cells reflect its effects on the polyamine metabolic pathway, intracellular SL11144 accumulation, polyamine pools (Put, Spd, and Spm), and regulatory enzyme (SSAT and ODC) activities

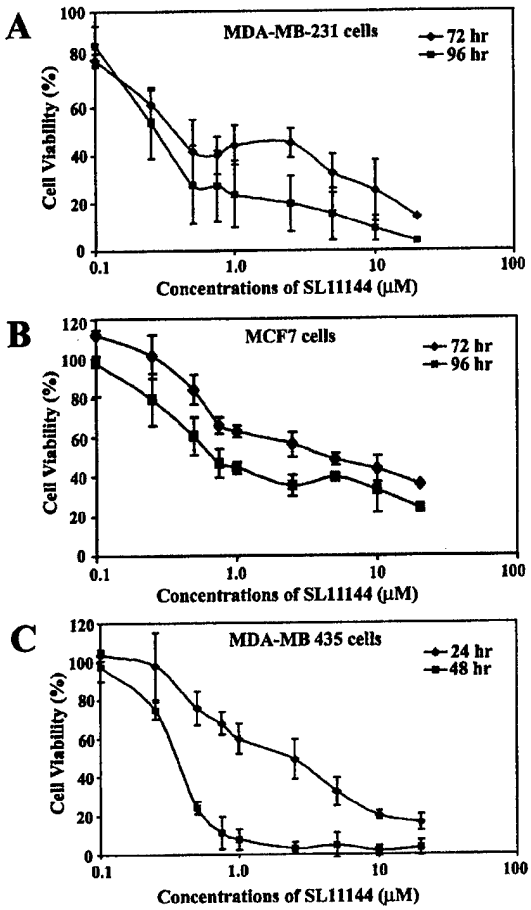

Fig. 2 SL11144 inhibits growth of human breast cancer cells in a time- and dose-dependent manner. MDA-MB-231 cells (*A*) and MCF7 cells (*B*) were treated with increasing concentrations of SL11144 for 72 or 96 h. MDA-MB-435 cells (*C*) were treated with increasing concentrations of SL11144 for 24 or 48 h. MTT assays were performed as described in "Materials and Methods." Shown are means ± SD of independent experiments performed in quadruplicate.

were assessed. As shown in Table 1, the accumulation rates of SL11144 in the three cell lines are similar after exposure of cells to 10 μM SL11144 for 24 h. SL11144 reduced all intracellular polyamines in MDA-MB-231 cells. Put and Spm were decreased, and Spd was slightly increased by SL11144 in MCF7 cells. In MDA-MB-435 cells, Spd level was down-regulated, and Spm was slightly up-regulated by SL11144. ODC activity was significantly inhibited, and SSAT activity was modestly increased in all three cell lines.

SL11144 Induces Apoptotic Cell Death. To determine whether observed SL11144-induced decrease in growth rate was a result of apoptosis, DNA fragmentation assays were performed. DNA ladders isolated from untreated and SL11144-treated cells were processed by agarose gel electrophoresis to detect the typical oligonucleosomal DNA fragmentation. The results (Fig. 3) indicate that SL11144 induces DNA fragmentation in all three human breast cancer cell lines, but the time and dose required for the induction of apoptosis varied by cell type.

Table 1 Effects of SL11144 on polyamine pools and SSAT and ODC activities in human breast cancer cells

Intracellular polyamine levels, SSAT activity, and ODC activity were determined as described in "Materials and Methods" after incubation of tumor cells for 24 h in the presence or absence of 10 μM SL11144. Values represent the means of duplicate determinations.

Cell lines	Treatment	SL11144 (nmol/mg protein)	Polyamines (nmol/mg protein)			SSAT activity (pmol/mg protein/min)	ODC activity (pmol CO_2/mg protein/h)
			Put	Spd	Spm		
MDA-MB-231	Control	ND[a]	3.65	52.04	21.17	2.22	2290.98
	SL11144	2.32	ND	15.27	8.14	6.02	ND
MCF7	Control	ND	6.22	49.38	28.52	1.39	2359.10
	SL11144	4.81	0.95	58.53	16.58	14.72	6.53
MDA-MB-435	Control	ND	ND	22.03	3.24	2.73	558.79
	SL11144	3.62	ND	8.84	4.35	23.39	4.58

[a] ND, not detected.

DNA fragmentation was clearly detected only after a 96-h exposure to SL11144 in MDA-MB-231 and MCF7 cells but was detectable in 12 h in MDA-MB-435 cells. A minimum concentration of 5 μM is required for SL11144 to induce DNA fragmentation in MDA-MB-231 and MDA-MB-435 cells, but MCF7 cells are more sensitive in that DNA fragmentation was observed with 0.1–0.25 μM SL11144 for 96 h.

The effect of SL11144 on cell morphology was also investigated. Both control and SL11144-treated cells were stained with the fluorescent dye Hoechst 33258 and visualized by fluorescence microscopy. Typical morphological changes of apoptosis including chromatin condensation and nuclear fragmentation were observed in all three treated cell lines (Fig. 4*B*), but not in the untreated control cells (Fig. 4*A*). Taken together, the DNA fragmentation and fluorescence results suggest that SL11144 induces apoptotic cell death in all three human breast cancer cell lines.

Therapeutic Effect of SL11144 against Human Breast Cancer MDA-MB-231 Xenografts. The *in vivo* therapeutic effect of SL11144 was evaluated using human breast cancer MDA-MB-231 xenografts in athymic nude mice. By 10 days after tumor cell inoculation, the average tumor sizes reached approximately 50–100 mm^3. Mice were then randomized into treatment ($n = 7$) and control ($n = 8$) groups. Different doses of SL11144 (2.5, 5, and 10 mg/kg) were administered via i.p. injections twice a week. SL11144 displayed antiproliferative effects against MDA-MB-231 xenografts in a dose-dependent manner (Fig. 5*A*). Whereas partial suppressions of tumor growth were observed at the doses of 2.5 and 5 mg/kg, increasing the dose to 10 mg/kg significantly inhibited the tumor growth. During the course of treatment, there was no obvious weight loss observed, except a slight decrease of weight (~5% of body weight) in the group treated with a dose of 10 mg/kg at 41 days (Fig. 5*B*). These results indicate that the treatment of SL11144 possesses significant *in vivo* growth suppression efficacy against MDA-MB-231 cells with no overt toxic effects.

Effects of SL11144 on Apoptosis-related Proteins. Several apoptosis-associated genes or proteins have been shown to play critical roles in regulating apoptosis. These include caspases, bcl-2 family members, FasL, cytochrome *c*, and PARP (25–29). To determine whether these proteins are involved in the mediation of SL11144-induced cell death in human breast cancer cells, we examined their expression by Western blotting. In MDA-MB-231 cells, as shown in Fig. 6*A*, treatment with 10 μM SL11144 decreased the amount of caspase-3 protein by 96 h of treatment, but no cleaved, active caspase-3 or its downstream target, PARP, was detected. We next examined whether two upstream proteases, caspase-9 and caspase-8, were affected by SL11144 in MDA-MB-231 cells. SL11144 treatment increased the cleavage of caspase-9 after 72 h, whereas caspase-8 was essentially undetectable under the conditions examined. Cytochrome *c* release from mitochondria was enhanced at 48 h and returned to its baseline level thereafter. Furthermore, Bcl-2 protein was down-regulated, and Bax was up-regulated by SL11144 beginning at 24 h. Finally, expression of FasL was increased by SL11144 after 48 h. The changes observed precede DNA fragmentation, which was not observed until 96 h of treatment.

Effects of SL11144 on apoptotic protein expression were further assessed in MCF7 cells. Our results (Fig. 6*B*) confirmed the previous finding (30) that caspase-3 is not expressed in MCF7 cells. The activities of caspase-8, caspase-9, and PARP were not affected by SL11144. SL11144 did not change Bcl-2 protein levels, whereas Bax expression was minimal. No release of cytochrome *c* was observed in SL11144-treated MCF7 cells. However, FasL level was increased by SL11144 after 48 h of treatment. These data suggest that SL11144 may induce apoptosis in MCF7 cells through caspase- and cytochrome *c* release-independent pathways. Another possibility is that the DNA fragmentation and apoptotic morphological changes noted in MCF7 cells are induced directly by SL11144. Many polyamine analogs bind strongly to DNA and are capable of inducing structural changes in chromatin (6).

In contrast to the above-mentioned results in MCF7 and MDA-MB-231 cells, SL11144 treatment of MDA-MB-435 cells led to caspase-3 activation and cleavage of PARP within 12 h of drug exposure (Fig. 6*C*). Also, caspase-8 was activated, and the proform of caspase-9 was completely cleaved by 48 h of exposure. Although Bcl-2 expression did not change, and Bax expression was essentially undetectable, cytochrome *c* was released into cytoplasm from mitochondria by 12 h. FasL expression was induced at 48 h. These results suggest that both caspase and mitochondrial pathways are activated by SL11144 in MDA-MB-435 cells. The time course of caspase activation and cytochrome *c* release parallels the course of DNA fragmentation, which was detected at 12 h of SL11144 treatment.

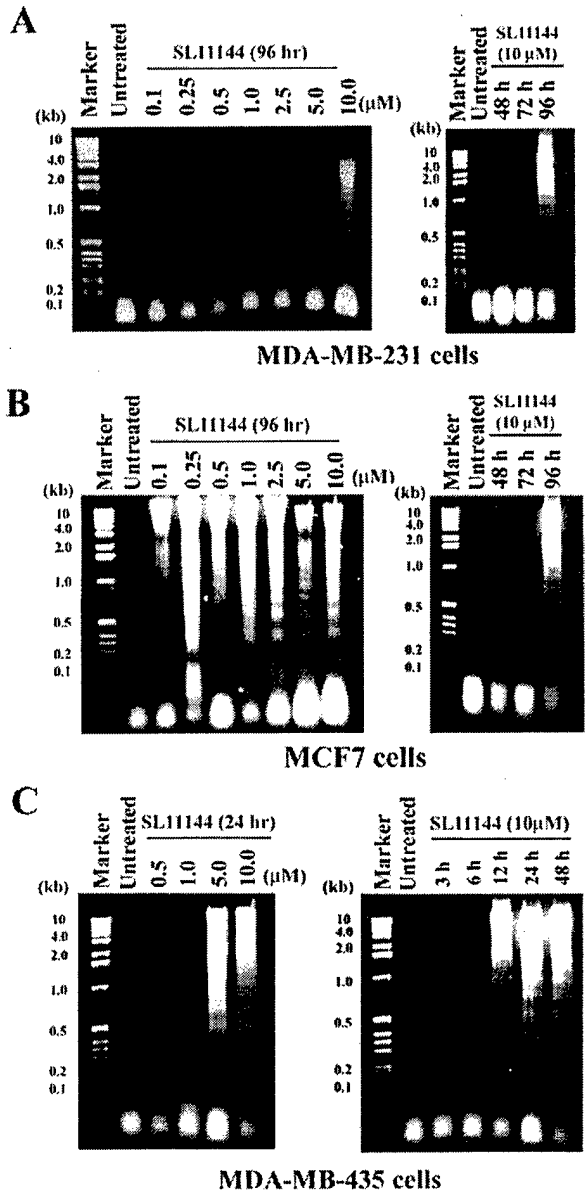

Fig. 3 SL11144 induces internucleosomal DNA fragmentation. MDA-MB-231 cells (*A*) and MCF7 cells (*B*) were treated with increasing concentrations of SL11144 (0.1–10 μM) for 96 h or treated with 10 μM SL11144 for 48, 72, and 96 h. MDA-MB-435 cells (*C*) were treated with increasing concentrations of SL11144 (0.1–10 μM) for 24 h or treated with 10 μM SL11144 for 3, 6, 12, 24, and 48 h. Cells were harvested, and fragmented DNA was extracted as described in "Materials and Methods." Fragmented DNA was analyzed by electrophoresis in a 1.2% agarose gel containing 0.1% ethidium bromide. Each experiment was done twice with similar results.

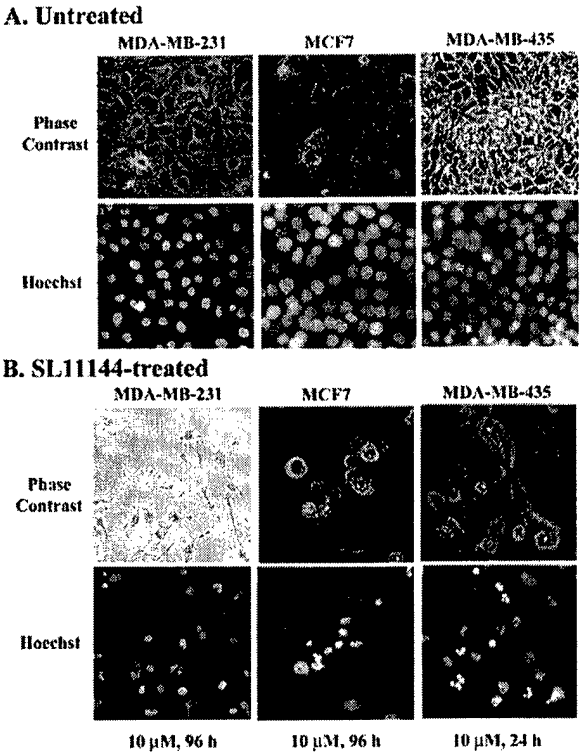

Fig. 4 Fluorescent micrographs of SL11144-treated cells. MDA-MB-231 cells, MDA-MCF7 cells, and MB-435 cells were exposed to 10 μM SL11144 for the indicated times. Then cells were fixed in formaldehyde and stained with Hoechst dye 33258. *A*, untreated cells; *B*, SL11144-treated cells.

SL11144 Up-Regulates c-Jun and c-Fos in MDA-MB-231 and MDA-MB-435 Cells. Because the effects of SL11144 on apoptotic pathways varied greatly between different human breast cancer cell lines, we examined the impact of SL11144 on other important apoptosis-related factors, particularly c-Jun and c-Fos. Both c-Jun and c-Fos are important members of the AP-1 transcription factor family, which plays a critical role in regulating transcription of a variety of genes involved in growth, differentiation, apoptosis, and so forth. In MDA-MB-231 cells, SL11144 treatment induced c-Jun phosphorylation after 48 h but did not alter the protein expression of either c-Jun or c-Fos (Fig. 7*A*). In MCF7 cells, no obvious changes in c-Jun and c-Fos were observed (Fig. 7*B*). In contrast, SL11144 significantly induced the phosphorylation of c-Jun and enhanced the protein level of c-Jun and c-Fos in MDA-MB-435 cells within 6–12 h (Fig. 7*C*). To study whether SL11144-enhanced c-Jun and c-Fos protein expression led to the increased nuclear localization of these proteins in MDA-MB-435 cells, the subcellular localization of c-Jun and c-Fos was examined. c-Jun was induced and expressed largely in the nucleus, whereas c-Fos was induced in both the cytoplasm and the nucleus (Fig. 7*D*). These results imply that up-regulations of AP-1 family proteins by SL11144 are cell type specific and may play an active role in the mediation of growth-inhibitory activities of SL11144.

DISCUSSION

Results of previous studies from our laboratory demonstrated that first and second generations of *N*-acetyl substituted polyamine analogs could inhibit growth and induce apoptosis in

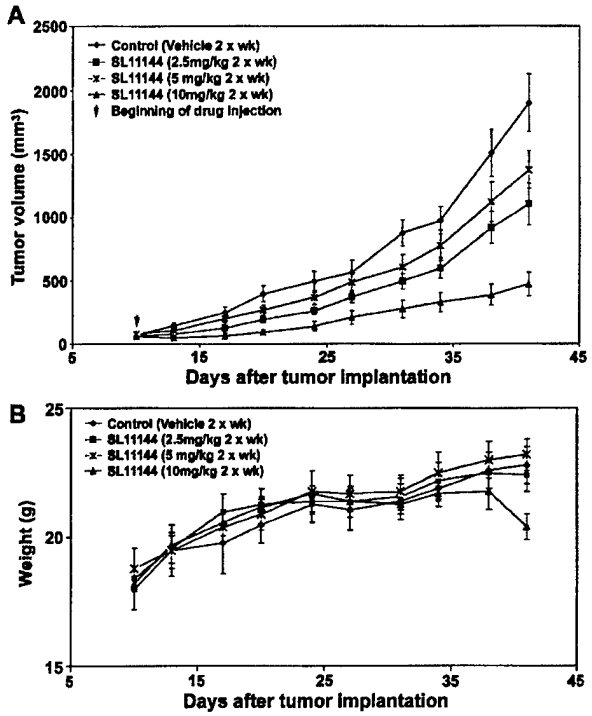

Fig. 5 Effects of SL11144 in nude mice bearing MDA-MB-231 xenografts. *A*, MDA-MB-231 cells were transplanted into the flank region of nude mice. Ten days after implantation, different doses of SL11144 (2.5, 5, or 10 mg/kg) or vehicle were given via i.p. injection twice weekly. Tumor volumes of mice were measured twice weekly. The *vertical bars* indicate mean tumor size (in mm^3) ± SE. *B*, weights of mice were measured twice weekly. The *vertical bars* indicate mean mouse weight (in g) ± SE.

MCF7 and other human breast cancer cell lines (3, 9, 31). However a Phase II clinical trial of one early polyamine analog, N^1,N^{11}-diethylnorspermine (DENSPM; also known as BENSpm), showed that it was not effective as a single agent in women with advanced breast cancer (32). Recently, a group of new polyamine analogs designated as oligoamines has been developed (17). Oligoamines were synthesized with longer chains than natural cellular polyamine molecules that occur in mammalian cells and are effective against a variety of proliferating cells (17). In this study, we demonstrate that one of the leading oligoamine compounds, SL11144, significantly inhibits the growth of and induces PCD in human breast cancer cells. It displays more potent antiproliferative activity against breast tumor line proliferation than the previously reported polyamine analogs, BENSpm, CPENSpm, and CHENSpm. SL11144 induced DNA fragmentation and typical apoptotic morphological changes in both hormone-responsive (MCF7) and hormone-unresponsive (MDA-MB-231 and MDA-MB-435) breast cancer cell lines. It appears that there is no relationship between hormone receptor status and cytotoxic effects of SL11144. SL11144 also inhibits the growth of MDA-MB-231 xenografts in nude mice in a dose-dependent fashion without apparent toxicity.

SL11144-induced apoptosis, based on morphological and DNA fragmentation criteria, was not detected until 96 h of treatment in MDA-MB-231 and in MCF7 cells, but 12 h of treatment with SL11144 resulted in apoptosis in MDA-MB-435 cells. Although the mechanisms of differential susceptibility among tumor cells to polyamine analog-induced cell death are unclear, this could reflect varied effects on apoptotic pathway members including caspases, bcl-2 family members, cytochrome *c*, and FasL, which have been demonstrated to play critical roles in regulating PCD (25–28). Caspases have been characterized as the effectors and executioners of apoptosis, and caspase-3 is a critical downstream apoptotic effector that cleaves specific substrates such as PARP. The observation that caspase-3 activation was followed by PARP cleavage in MDA-MB-435 cells indicates that caspase-3 may play a key role as an important executioner in SL11144-induced apoptosis in this cell line. However, the failure of SL11144 to activate caspase-3 in MDA-MB-231 cells and the absence of caspase-3 expression in MCF7 suggest that other factors or pathways can also function as apoptotic effectors in these two cell lines.

Mitochondria can be induced to release cytochrome *c* in response to many anticancer drugs and to other stresses by the opening of channels on the outer mitochondrial membrane (33). Release of cytochrome *c* activates the caspase adaptor, caspase-9, which then activates downstream caspases such as caspase-3 and caspase-8 (34). Our studies found that cytochrome *c* release was transiently enhanced by SL11144 with a 48-h drug exposure in MDA-MB-231 cells, whereas it was rapidly and consistently induced in MDA-MB-435 cells. In both cell lines, time-dependent activation of caspase-9 was observed, but caspase-8 activation was only seen in MDA-MB-435 cells. However, in MCF7 cells, SL11144 has no effect on cytochrome *c* release or on caspase-8 or -9 activation. The simultaneous activation of both caspase cascades and of the mitochondrial pathway in MDA-MB-435 cells by SL11144 might explain why cell death was more rapidly induced in these cells than in MDA-MB-231 or MCF7 cells. A recent study by Ellison *et al.* (35) reported that MDA-MB-435 cells might be of melanoma origin based on differential expressions of genes characteristic of breast cancer cells. If more compelling evidence ultimately confirmed this conclusion, the different sensitivity of MDA-MB-435 cells to SL11144 may possibly reflect its non-breast cancer identity.

Members of the Bcl-2 family play a central role in regulating the mitochondrial pathway of apoptosis. More than 20 Bcl-2 family members have been identified to date, including antiapoptosis members (Bcl-2, Bcl-X_L, Bcl-W, Bcl-G, Mcl-1, and so forth) and proapoptosis members [Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-Xs, and so forth (33, 34, 36–39)]. In response to various stimuli and stresses, Bcl-2 family proteins usually translocate to the outer mitochondrial membrane and modulate membrane permeabilization, leading to the release of cytochrome *c*. SL11144 decreased Bcl-2 and increased Bax expression in MDA-MB-231 cells but did not affect Bcl-2 and Bax in MDA-MB-435 and MCF7 cells, suggesting that the regulation of Bcl-2 family members by polyamine analog is cell type specific. Our data also demonstrate that SL11144 enhances FasL (the only protein to be uniformly affected) expression in all three cell lines. The Fas/FasL (CD95-CD95 ligand) system is another critical pathway that leads to the activation of apoptotic

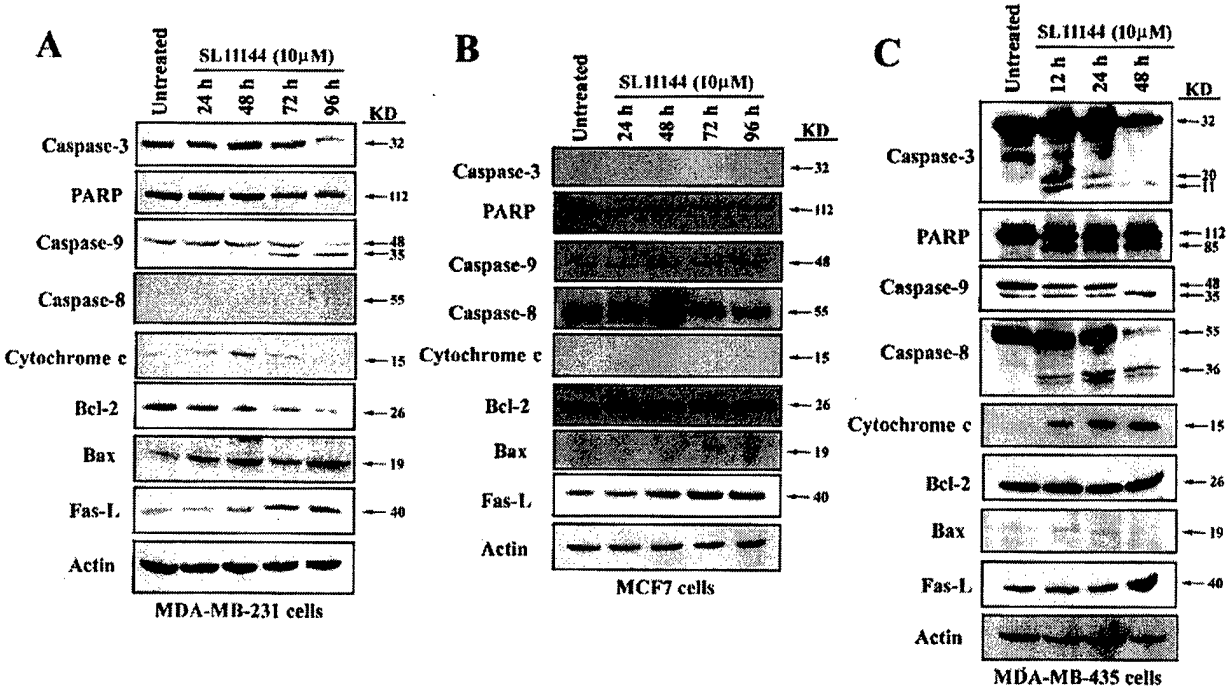

Fig. 6 Effects of SL11144 on apoptosis proteins. MDA-MB-231 cells (*A*) and MCF7 cells (*B*) were treated with 10 μM SL11144 for 24, 48, 72, and 96 h. MDA-MB-435 cells (*C*) were treated 10 μM SL11144 for 12, 24, and 48 h. Equal amounts (50 μg/lane) of cellular protein were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-caspase-3, PARP, caspase-9, caspase-8, cytochrome *c*, Bcl-2, Bax, or FasL monoclonal or polyclonal antibodies and analyzed as described in "Materials and Methods." Actin protein was blotted as a control. Each experiment was repeated twice with similar results.

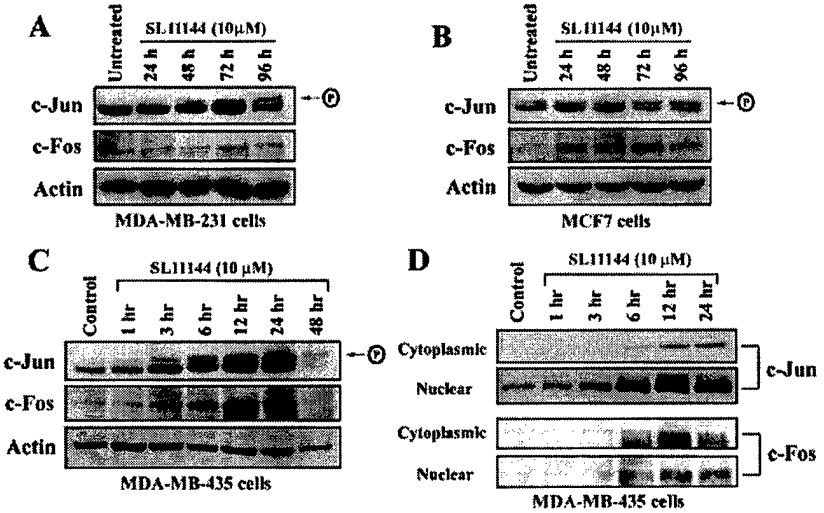

Fig. 7 Effects of SL11144 on c-Jun and c-Fos. Tumor cells were treated with 10 μM SL11144 for the times indicated. Equal amounts (50 μg/lane) of whole cell (*A*–*C*), cytoplasmic or nuclear protein (*D*) were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-c-Jun and c-Fos polyclonal antibodies and analyzed as described in "Materials and Methods." Actin protein was blotted as a control.

machinery. Binding of FasL to Fas and to other death receptors results in receptor trimerization, recruitment of adaptor protein to the cytoplasmic death domain, and activation of a series of downstream apoptotic events (40, 41). Recent studies have shown that overexpression of FasL can lead to suicidal or fratricidal destruction in melanoma and leukemia cells via autocrine or fratricidal interactions between FasL and Fas (42, 43). Up-regulation of FasL level by SL11144 in all three human breast cancer cell lines implies that activation of Fas/FasL system might be a common mechanism for the cell death induced by SL11144.

We further investigated whether other important upstream

regulatory or signaling events were involved in the mediation of SL11144-induced growth inhibition and apoptosis. SL11144 induces expression and phosphorylation of c-Jun, an important member of the AP-1 family, in both MDA-MB-231 and MDA-MB-435 cells. It also significantly increased the protein expression of another important AP-1 family member, c-Fos, after 12 h in MDA-MB-435 cells. Nuclear extraction analysis showed that c-Jun protein was located largely in the nucleus, where it can potentially play an active role in mediation of a wide range of gene expressions. However, neither c-Jun or c-Fos levels nor phosphorylation status was significantly affected by SL11144 treatment in MCF7 cells. c-Jun-NH_2-terminal kinase signaling and AP-1 transcription factors have been implicated in the regulation of cell proliferation, differentiation, and apoptosis (44). The proapoptotic targets of c-jun include FasL, tumor necrosis factor α, c-Myc, p53, and members of the bcl-2 family (45–49). The activation of c-Jun in MDA-MB-231 and MDA-MB-435 cells, but not in MCF7 cells, suggests that c-Jun-NH_2-terminal kinase/AP-1 and the upstream regulator mitogen-activated protein kinase family might be a major polyamine analog response pathway in some but not all breast cancer cell lines.

The intracellular polyamines are highly regulated by several polyamine metabolic enzymes. ODC, the first and rate-limiting step of polyamine biosynthesis, increases levels of polyamines in cells during rapid proliferation or differentiation (6). High expression of ODC characterizes some cancers including breast cancer. As a result, there has been extensive effort to design compounds that can inhibit ODC activity in tumor cells. α-Difluoromethylornithine, an irreversible inhibitor of ODC, has proven to be effective in inhibiting growth in several *in vitro* and *in vivo* tumor models (6, 50). In this study, the effect of SL11144 on natural polyamine levels was variable in different cell lines. SL11144 treatment led to a decrease in all natural polyamines in MDA-MB-231 cells and had inconsistent effects in MCF7 and MDA-MB-435 cells. The increased level of Spd in MCF7 cells by SL11144 might explain why MCF7 cells are less sensitive to SL11144 than the other two cell lines. Although ODC activities were significantly suppressed by SL11144 in all these cells, it is not clear whether the attenuation of ODC activities contributes to SL11144 cytotoxicity. In addition, the activity of another critical polyamine metabolic enzyme, SSAT, was only modestly up-regulated by SL11144 exposure, indicating that SSAT activity is not responsible for the observed cytotoxic response. All these results imply that the effects of SL11144 may not be solely a function of its effect on polyamine pools.

In summary, a newly developed polyamine analog, SL11144, exhibits significant inhibitory actions against human breast cancer cell growth *in vitro* and *in vivo*. Apoptotic cell death was induced by SL11144 in a time- and dose-dependent manner. SL11144 modulated expression of apoptotic proteins in a cell type-specific manner, suggesting that multiple apoptotic pathways might be involved in SL1144-induced apoptosis in different human breast cancer cell lines.

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Regulation of Polyamine Analogue Cytotoxicity by c-Jun in Human MDA-MB-435 Cancer Cells

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Abstract

Several polyamine analogues have efficacy against a variety of epithelial tumor models including breast cancer. Recently, a novel class of polyamine analogues designated as oligoamines has been developed. Here, we demonstrate that several representative oligoamine compounds inhibit *in vitro* growth of human breast cancer MDA-MB-435 cells. The activator protein-1 (AP-1) transcriptional factor family members, c-Jun and c-Fos, are up-regulated by oligoamines in MDA-MB-435 cells, suggesting a possible AP-1-dependent induction of apoptosis. However, the use of a novel c-Jun NH_2-terminal kinase (JNK) inhibitor, SP600125, suggests that inhibition of c-Jun activity sensitized tumor cells to oligoamine-induced cell death. To directly test this hypothesis, cells were stably transfected with the dominant-negative mutant c-Jun (TAM67), which lacks the NH_2-terminal transactivation domain. Cells overexpressing TAM67 exhibit normal growth kinetics but demonstrate a significantly increased sensitivity to oligoamine cytotoxicity and attenuated colony formation after oligoamine treatment. Furthermore, oligoamine treatment leads to more profound caspase-3 activation and poly(ADP-ribose) polymerase cleavage in TAM67 transfectants, suggesting that c-Jun acts as an antiapoptosis factor in MDA-MB-435 cells in response to oligoamine treatment. These findings indicate that oligoamine-inducible AP-1 plays a prosurvival role in oligoamine-treated MDA-MB-435 cells and that JNK/AP-1 might be a potential target for enhancing the therapeutic efficacy of polyamine analogues in human breast cancer.

Introduction

The polyamines (putrescine, spermidine, and spermine) are naturally occurring polycationic alkylamines that are required for cell growth. Because of the critical role of polyamines in the regulation of cell growth, the polyamine metabolic pathway is an attractive target for antineoplastic strategies (1–3). The primary regulatory enzymes of polyamine biosynthesis include ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase, spermidine synthase, and spermine synthase. The enzymes spermidine/spermine N^1-acetyltransferase (SSAT) and spermine oxidase also play a significant rate-limiting role in polyamine catabolism (4–6). The best characterized polyamine biosynthetic pathway inhibitor is α-difluoromethylornithine, an irreversible inhibitor of ODC, which inhibits tumor cell growth in preclinical models, has been tested in phase I/II clinical trials, and is currently being examined as a chemopreventive agent (7–9).

Based on the feedback mechanisms of natural polyamines, whereby they regulate their own synthesis, another useful strategy to develop polyamine metabolism inhibitors is the use of polyamine analogues that can mimic some of the regulatory roles of natural polyamines but are unable to replace the actual function of natural polyamines for cell growth (10–12). Several polyamine analogues have shown antineoplastic activity in a variety of tumor types. For example, N^1, N^{11}-diethylnorspermine inhibits polyamine biosynthesis, decreases polyamine transport, and greatly increases SSAT activity in several tumor models (13, 14). Recently, a series of novel oligoamine analogues have been synthesized (15). Some of these new analogues have shown significant inhibitory activity against *in vitro* and *in vivo* tumor cell growth (16, 17).

In our previous studies, several early polyamine analogues were reported to induce programmed cell death in human breast cancer cells, but the cell death mechanisms have been uncertain (18). For example, N^1,N^{11}-diethylnorspermine-related superinduction of SSAT with subsequent depletion of natural polyamine pools was noted in specific human breast cancer cell lines (19), a finding that has been confirmed by conditional overexpression of SSAT in breast cancer MCF-7 cells (20). Although superinduction of SSAT seems to be directly associated with growth inhibition by some polyamine analogues, other polyamine analogues do not highly induce SSAT but can still inhibit tumor cell growth (21, 22). These results suggest that polyamine analogue-induced cell death occurs through multiple agent-specific and cell type-specific mechanisms. Several important growth-associated or cell cycle-associated genes or pathways have been reported to be affected by specific polyamine analogues (17, 23, 24). However, little is known thus far about the exact mechanisms by which other polyamine analogues inhibit growth and induce apoptosis in tumor cells.

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An important mediator that is widely involved in regulating cellular proliferation, differentiation, and apoptosis is the activator protein-1 (AP-1) transcription factor family (25). AP-1 proteins are homodimers or heterodimers composed of leucine zipper proteins that belong to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun dimerization partners (JDP1 and JDP2), and activating transcription factors (ATF2, LRF1/ATF3, and B-ATF) subgroups (26, 27). The major upstream regulator of AP-1 is the c-Jun NH_2-terminal kinase (JNK), one subgroup of the mitogen-activated protein kinase (MAPK) family. After exposure of cells to several cytokines or extracellular stresses, JNK is activated and subsequently phosphorylates its major target, c-Jun, and related molecules. This in turn leads to the enhanced transcriptional regulation of c-Jun and its dimerization partners with subsequent effects on the promoter regions of downstream target genes (27).

In our previous work, we noted that a new oligoamine analogue, SL-11144, inhibited growth and induced apoptosis in several human breast cancer cell lines (17). In some cases, this was associated with induction of the Jun protein. In this study, we have examined the mechanisms of cytotoxicity of several oligoamines using the human MDA-MB-435 cancer cell line as a model system. Our data demonstrate that these oligoamines significantly inhibit cell growth and activate AP-1 members, c-Jun and c-Fos. By using a selective JNK inhibitor and the stable transfection of dominant-negative mutant c-Jun (TAM67), we provide evidence that c-Jun plays a protective role in oligoamine-induced cell death in MDA-MB-435 cells.

Results

Growth Inhibition by Oligoamines

The MDA-MB-435 cells were chosen as a model system as they exhibit highly metastatic and aggressive behavior *in vivo* compared with other human breast cancer cell lines (28–30). The *in vitro* sensitivities of MDA-MB-435 cells to SL-11144, SL-11159, and SL-11172 (Fig. 1) were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth inhibition assay. Tumor cells were treated with increasing concentrations of oligoamines for 24 and 48 h, respectively. All three agents showed significant growth inhibitory effects against MDA-MB-435 cells in a time- and dose-dependent manner (Fig. 2).

Oligoamines Up-Regulate c-Jun and c-Fos

AP-1 is an important signaling complex in the regulation of cellular proliferation, differentiation, apoptosis, and metastasis (31). Therefore, we first examined the impact of oligoamines on the expression of AP-1 family members, c-Jun and c-Fos, in MDA-MB-435 cells. All three oligoamines significantly induced the phosphorylation of c-Jun and enhanced the protein level of both c-Jun and c-Fos in a dose-dependent manner within 24 h of treatment (Fig. 3). The phosphorylation of c-Jun can occur within 6 h of oligoamine exposure (data not shown). These results suggested that up-regulation of the AP-1 family might play an active role in the mediation of oligoamine-induced growth inhibition in MDA-MB-435 cells.

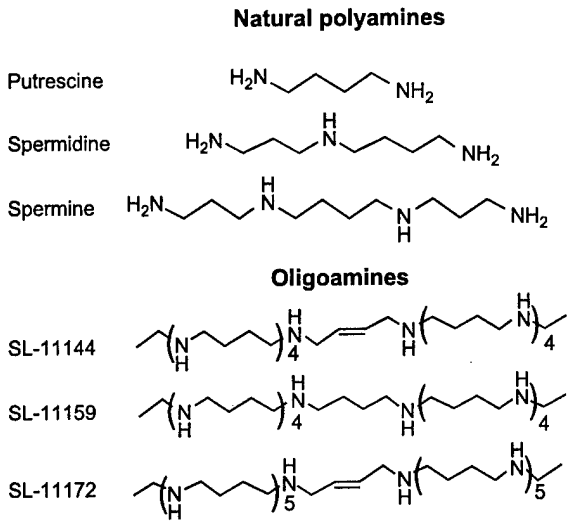

FIGURE 1. Structures of natural polyamines and oligoamines SL-11144, SL-11159, and SL-11172.

SP600125 Blocks c-Jun Activation and Promotes the Cytotoxicity of Oligoamine

SP600125 is a novel selective JNK1, JNK2, and JNK3 inhibitor that can inhibit the phosphorylation of c-Jun (32, 33). To examine whether the inhibition of c-Jun phosphorylation could affect oligoamine-induced cell death, MDA-MB-435 cells were treated with SP600125 or oligoamines alone or simultaneously. By Western blot, we determined that cotreatment with oligoamines and SP600125 significantly blocked the phosphorylation and decreased the expression of c-Jun compared with that seen with oligoamine treatment alone (Fig. 4A). MTT analysis of the effects of the c-Jun phosphorylation inhibitor on oligoamine-induced cell growth inhibition indicated that simultaneous treatment with SP600125 and oligoamine resulted in increased cytotoxicity compared with that seen with either agent alone (Fig. 4B). It is important to note that SP600125 treatment does not affect accumulation of SL-11144 in MDA-MB-435 cells (data not shown).

Overexpression of TAM67 Sensitizes Tumor Cells to Oligoamine Cytotoxicity

The finding that SP600125 blocks JNK/c-Jun pathway activation and increases the cytotoxicity of oligoamines suggests that the transcription factor c-Jun may exert a protective role in oligoamine-induced cell death. However, further evidence is needed to support this hypothesis because JNK activation may concurrently regulate a variety of other downstream genes or pathways with diverse functions (27). To further dissect the effect of c-Jun activity on oligoamine-induced cell death, a vector expressing the c-Jun dominant-negative mutant, TAM67, was constructed and stably transfected into MDA-MB-435 cells. Wild-type c-Jun can be phosphorylated by JNK at two serine residues (Ser^{63} and Ser^{73}) proximal to the transactivation domain, which is required for the efficient transactivation of c-Jun (34, 35). TAM67 is a mutant form of c-Jun in which the transactivation domain (amino acids 3–122)

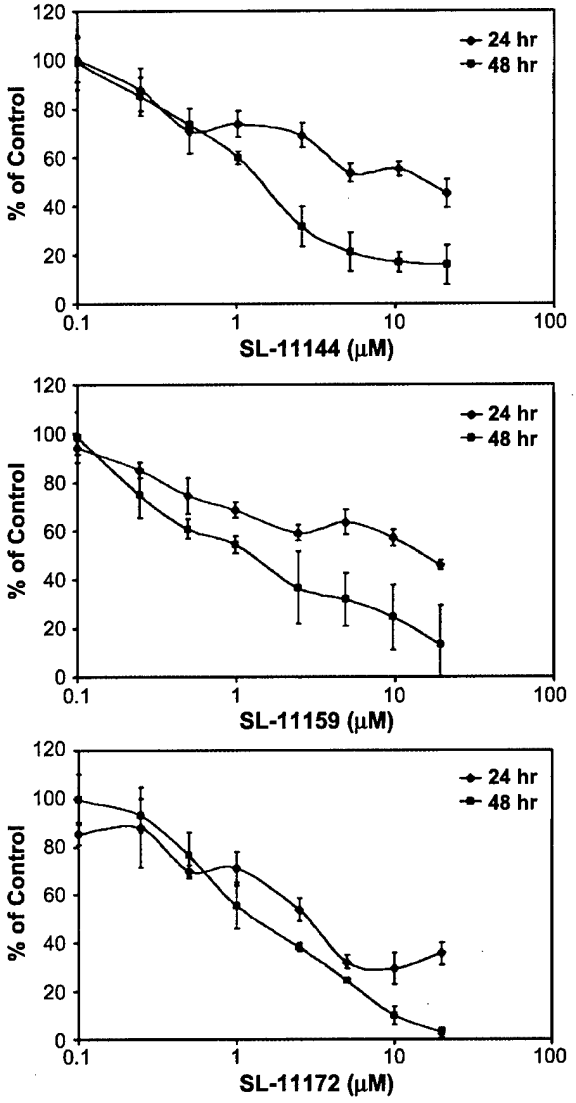

FIGURE 2. Oligoamines inhibit growth of human breast cancer MDA-MB-435 cells in a time- and dose-dependent manner. MDA-MB-435 cells were treated with increasing concentrations of SL-11144, SL-11159, or SL-11172 for 24 or 48 h. MTT assays were performed as described in "Materials and Methods." *Points,* means of three independent experiments performed in quadruplicate; *bars,* SD.

has been deleted, leaving the COOH-terminal DNA binding and dimerization domain intact. Such a mutant c-Jun protein is unable to activate its target genes but still possesses the ability to bind to AP-1 site in promoter regions of target genes and competitively quench the transactivation activity of endogenous wild-type Jun and its dimerization partners (36, 37). Three single clones (TAM67-12, TAM67-19, and TAM67-21) that highly express the ~29-kDa dominant-negative mutant c-Jun protein were identified (Fig. 5A), and these three clones were used for a series of functional experiments. We first examined the effect of TAM67 on the general growth rate of MDA-MB-435 transfectants. Cell growth over 96 h was similar for the parental wild-type, vector, and TAM67 mutant cell lines (Fig. 5B), indicating that the stable expression of either empty vector or TAM67 does not adversely affect the growth of MDA-MB-435 cells.

TAM67 transfected clones were then compared to determine whether the expression of the dominant-negative mutant c-Jun altered the response of tumor cells to oligoamines. The vector control clone and the three independent dominant-negative mutant c-Jun transfectants were treated with increasing concentrations of SL-11144, SL-11159, or SL-11172 (1–20 μM) for 24 h and analyzed by MTT. When compared with the vector control, all three TAM67 clones were significantly more sensitive to treatment with SL-11144 and SL-11159 at all doses studied ($P < 0.001$). The TAM67 transfectants were also more sensitive to lower concentrations of SL-11172 treatment (1–2.5 μM, $P < 0.001$; Fig. 6). However, at higher concentrations (5.0 μM or higher) of SL-11172 treatment, there was no obvious difference in cellular sensitivity to SL1-11172 between controls and transfectants.

To confirm the increased sensitivity of TAM67 transfectants to oligoamines, vector and TAM67 transfected cells were subjected to colony formation analysis. Cells were treated with 0.1 μM of each oligoamine for 12 h. The colony-forming efficiency of all three TAM67 transfected cell lines was dramatically diminished after treatment with all three oligoamines compared with the vector transfectant (Fig. 7). These results suggest that specific inhibition of transactivation of endogenous wild-type c-Jun significantly increases the sensitivity of MDA-MB-435 cells to oligoamine cytotoxicity.

TAM67 Overexpression Enhances Oligoamine-Induced Apoptosis

In our recent studies, oligoamines have been demonstrated to activate apoptosis-related pathways and induce apoptotic cell death in human breast cancer cells (17). To investigate if overexpression of dominant-negative mutant c-Jun could affect apoptotic response to oligoamines in MDA-MB-435 cells, expression of two key apoptosis effectors, caspase-3 and poly(ADP-ribose) polymerase (PARP), was examined by Western blotting. The vector transfectant and a representative dominant-negative mutant c-Jun transfectant (TAM67-21) were

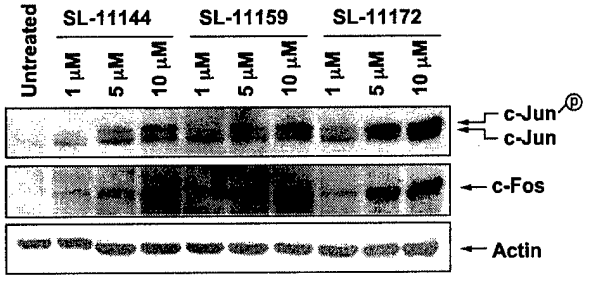

FIGURE 3. Effects of oligoamines on c-Jun and c-Fos. MDA-MB-435 cells were treated with 1, 5, and 10 μM of SL-11144, SL-11159, or SL-11172 for 24 h. Equal amounts of protein (50 μg/lane) were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-c-Jun and anti-c-Fos polyclonal antibodies and analysis as described in "Materials and Methods." Actin protein was blotted as a control. Each experiment was repeated twice with similar results.

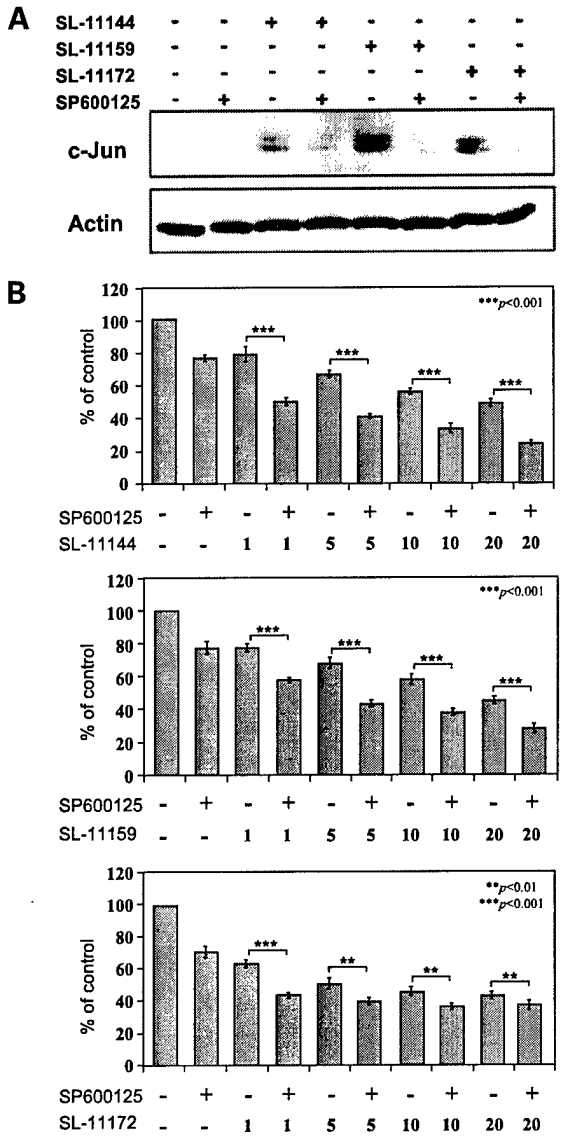

FIGURE 4. SP600125 blocks c-Jun activation and promotes the cytotoxicity of oligoamine. **A.** MDA-MB-435 cells were treated with 10 μM SL-11144, SL-11159, or SL-11172 with or without the cotreatment with 10 μM SP600125 for 24 h. Equal amounts of cellular protein (50 μg/lane) were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-c-Jun polyclonal antibodies and analysis as described in "Materials and Methods." Actin protein was blotted as a control. Each experiment was repeated twice with similar results. **B.** MDA-MB-435 cells were treated with 10 μM SL-11144, SL-11159, or SL-11172 with or without the cotreatment with 10 μM SP600125 for 24 h. MTT assays were performed as described in "Materials and Methods." *Columns,* means of three independent experiments performed in quadruplicate; *bars,* SD. **, $P < 0.01$; ***, $P < 0.001$, statistically significant differences using Student's *t* test.

treated with SL-11144 for 24 h. SL-11144 treatment led to a greater decrease in the caspase-3 level and more significant cleavage of the PARP protein in TAM67-21 cells compared with the vector control clone (Fig. 8). These results indicate that inhibition of c-Jun activation may enhance apoptotic cell death induced by oligoamines in MDA-MB-435 cells.

Effects of Oligoamine on Intracellular Polyamines Metabolism in TAM67 and Vector Control Transfectants

To address whether the overexpression of TAM67 alters the effects of oligoamines on the polyamine metabolic pathway, we evaluated the intracellular polyamine pools and the activities of ODC, a key polyamine biosynthetic enzyme, and SSAT, a key polyamine catabolic enzyme, in empty vector and TAM67 transfected cells. The TAM67-21 transfectant was again selected as representative for these studies. Treatment of both vector controls and TAM67-21 transfectants with 10 μM SL-11144 for 12 h was associated with a similar decrease in natural polyamine levels, increased SSAT activity, and diminished ODC activity in both cell lines (Table 1).

Discussion

The antiproliferative mechanisms of various polyamine analogues are being elucidated. Possible mechanisms include the fact that polyamine analogues can bind to nucleic acids, leading to the distortion of nucleic acid structure and impairment of normal function (38). Another potential

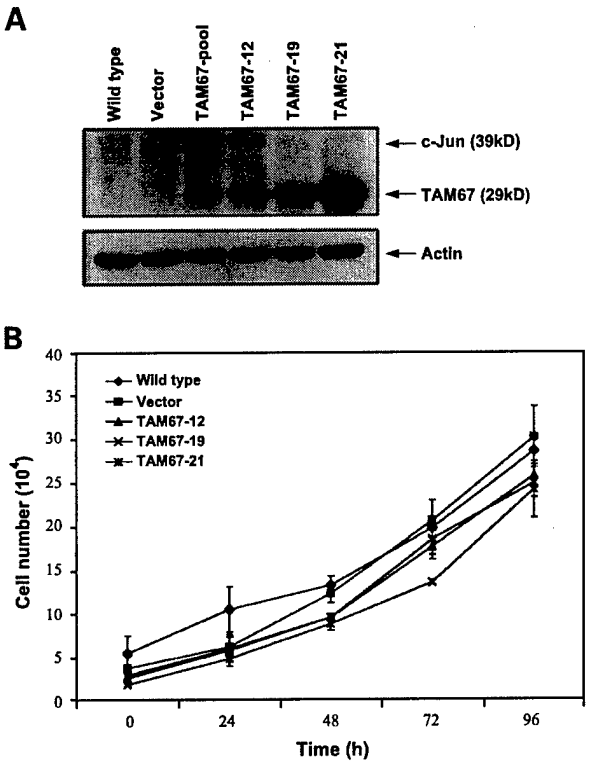

FIGURE 5. Characterization of TAM67 transfectants. **A.** MDA-MB-435 cells were stably transfected with pcDNA3.1-TAM67 as described in "Materials and Methods." Protein isolated from parental cells, empty vector transfectants, and TAM67 transfected single colonies were subjected to immunoblotting with an antibody to the COOH terminus of c-Jun to detect TAM67 at 29 kDa. Three geneticin-resistant clones (TAM67-12, TAM67-19, and TAM67-21) were shown to express high level of TAM67. Endogenous Jun proteins appeared at the *upper region* of the gel around 39 kDa. **B.** Proliferation rates of parental cells, empty vector transfectants, and three independent TAM67 stable transfectants were measured as described in "Materials and Methods." *Points,* averages of three independent experiments performed in triplicate; *bars,* SD.

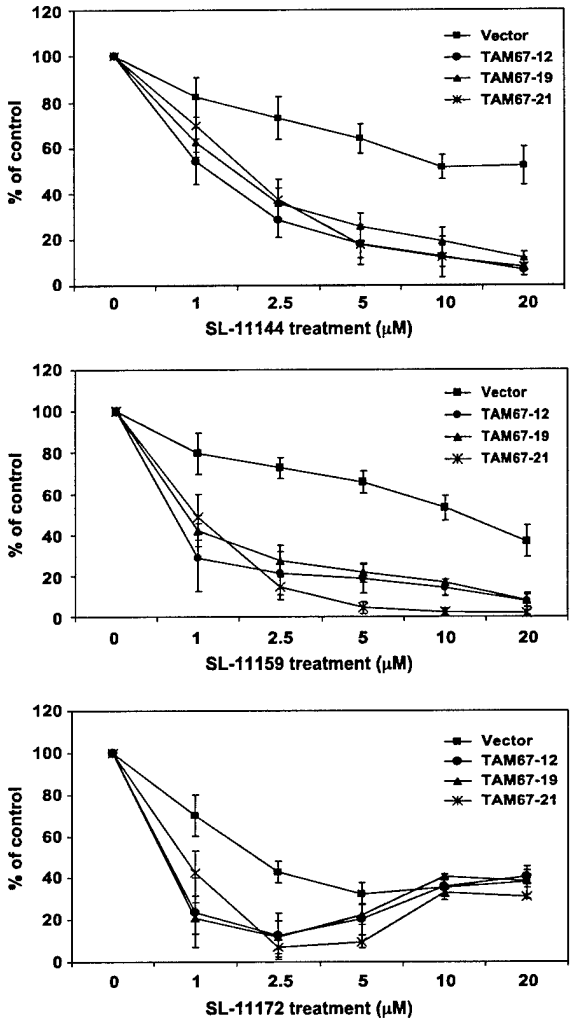

FIGURE 6. Overexpression of TAM67 sensitizes tumor cells to oligoamine cytotoxicity. Cells transfected with empty pcDNA3.1 vectors (*Vector*) and TAM67 cDNA transfected clones (*TAM67-12*, *TAM67-19*, and *TAM67-21*) were treated with 1, 2.5, 5, or 10 μM of SL-11144, SL-11159, and SL-11172 for 24 h. MTT assays were performed as described in "Materials and Methods." *Points,* means of three independent experiments performed in quadruplicate; *bars,* SD. $P < 0.001$, statistically significant differences using Student's *t* test between transfected cells and parental or vector transfected cells treated by SL-11144 (2.5–20 μM), SL-11159 (2.5–20 μM), and SL11172 (1.0 and 2.5 μM).

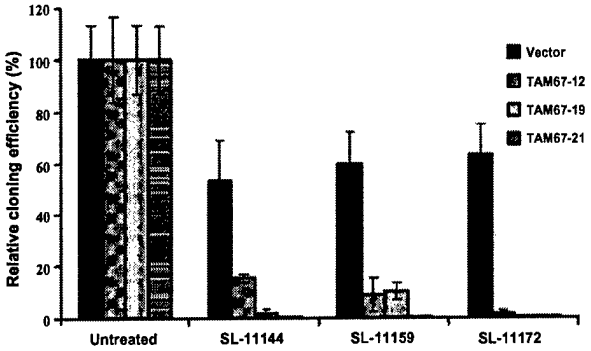

FIGURE 7. Effect of TAM67 transfection on colony formation. Vector and TAM67 transfected MDA-MB-435 cells were treated with 0.1 μM oligoamines (SL-11144, SL-11159, or SL-11172) for 12 h. Cells were washed, medium was replaced with drug-free medium, and cells were incubated for 8 days. Positive colonies were counted as described in "Materials and Methods." Results are average percentages of relative colony formation efficiency (numbers of colonies in treated groups/numbers of colonies in untreated groups). *Columns,* means of three independent experiments plated in triplicate; *bars,* SD.

mechanism is the rapid depletion of the natural polyamine pool associated with the superinduction of SSAT by polyamine analogues in some cell lines (19, 20, 22, 39). The oligoamines have longer chains than natural mammalian cellular polyamine molecules, which allow the analogues to condense and collapse DNA at much lower concentrations (15, 16).

In this work, we demonstrate that the decamines, SL-11144 and SL-11159, and the dodecamine, SL-11172, significantly inhibit the *in vitro* growth of human breast cancer MDA-MB-435 cells and increase c-Jun and c-Fos expression and c-Jun phosphorylation (Fig. 3). Cotreatment with a JNK selective inhibitor, SP600125, sensitizes tumor cells to the oligoamines.

This result implicates a possible protective role of the JNK/Jun pathway in oligoamine-treated MDA-MB-435 cells. AP-1 family members, particularly c-Jun and c-Fos, play critical roles in cellular proliferation, differentiation, transformation, and apoptosis, likely through their effects on several important AP-1 target genes, including cyclin D1, p16, $p19^{Arf}$, p53, $p21^{cip1/waf1}$, and Fas L (40–43). Due to the complexity of regulatory signaling impinging on AP-1/c-Jun, the net effect of AP-1 on the balance between cell survival and death varies greatly, depending on cell context, nature of the extracellular stimuli, and the signaling pathways that are simultaneously activated. For example, studies in neuronal cells demonstrated that inhibition of c-Jun activity by dominant-negative mutant

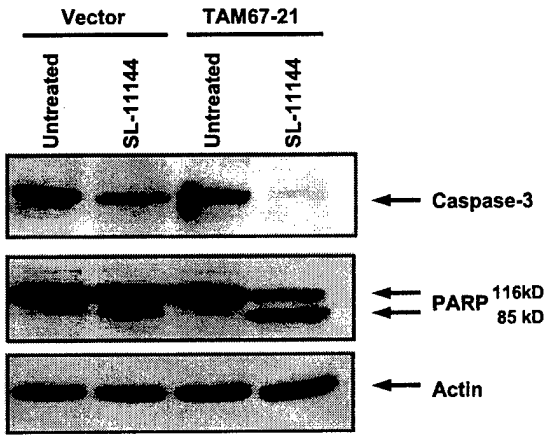

FIGURE 8. Effects of TAM67 on oligoamine-induced apoptosis. Parental, vector, and TAM67 transfected MDA-MB-435 cells were treated with 10 μM SL-11144 for 24 h. Equal amounts of protein (50 μg/lane) were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-caspase-3 and anti-PARP monoclonal or polyclonal antibodies and analysis as described in "Materials and Methods." Actin protein was blotted as a control. Each experiment was repeated twice with similar results.

Table 1. Effects of Oligoamine on Polyamine Pools, SSAT, and ODC Activities in Empty Vector and TAM67 Transfected Cells

Cell Lines	Treatment	Polyamines (nmol/mg protein)			SSAT Activity (pmol/mg/protein/min)	ODC Activity (pmol CO_2/mg protein/h)
		Putrescine	Spermidine	Spermine		
Vector	Control	0.56	19.50	22.44	7.93	460.81
	SL-11144	ND	5.12	7.21	82.42	3.59
TAM67-21	Control	ND	17.38	13.67	7.49	345.57
	SL-11144	ND	4.05	7.47	50.86	10.92

Note: Intracellular polyamine levels, SSAT activity, and ODC activity were determined as described in "Materials and Methods" following incubation of tumor cells for 12 h in the presence of 10 (μM SL-11144. Values represent the means of duplicated determinations. ND, not detected.

forms protects cells from apoptosis induced by withdrawal of nerve growth factor, and expression of a phosphorylation-deficient c-Jun mutant blocked phosphorylation of c-Jun by JNK and inhibited apoptosis (44–48). However, other lines of evidence suggest that JNK/Jun activation may protect other cells from stress-induced death. For example, *in vitro* studies demonstrated that JNK1 plays a protective role in Fas-induced apoptosis (49), and cells overexpressing mutant c-Jun are more vulnerable to apoptosis triggered by UV irradiation (50). Other *in vivo* studies suggested that c-Jun-deficient embryos exhibited massive apoptosis in liver cells (51). Taken together, these results suggest that AP-1 may have divergent functions in regulating stress-mediated cell death in different cell contexts.

To study the exact role of c-Jun in oligoamine-induced cell growth inhibition and death, clones of the MDA-MB-435 cell line that stably express a mutant transactivation domain deletion c-Jun (TAM67) were established. All three transfectants displayed normal growth, suggesting that dominant-negative mutant c-Jun protein is well tolerated in untreated cells. However, MTT proliferation assays show that mutant c-Jun transfectants were significantly more sensitive to the oligoamines over a broad range of concentrations. In addition, TAM67 transfectants exhibited dramatically reduced clonogenic efficiency following oligoamine exposure. These results indicate that c-Jun is a stress response protein and cell survival promoter in MDA-MB-435 cells. It is interesting to note that transfectants showed increased susceptibility to the dodecamine analogue, SL-11172, only with low-dose treatment (<5 μM). One possible explanation is that, structurally, SL-11172 has a longer chain than the decamines, SL-11144 and SL-11159, and therefore exhibits stronger DNA binding ability and cellular toxicity. Higher doses of SL-11172 induce more significant expression and phosphorylation of endogenous c-Jun than SL-11144 or SL-11159 (Fig. 3); thus, the level of TAM67 expression may not be sufficient to suppress the protective activities of endogenous c-Jun induced by higher doses of SL-11172. Previous studies in our laboratory demonstrated that oligoamines can induce apoptotic cell death in MDA-MB-435 cells through activation of critical death effectors like caspase-3 and PARP (17). The current study revealed that oligoamine produces a more profound caspase-3 activation and PARP cleavage in TAM67 transfectants than in control cells, suggesting that c-Jun acts as an antiapoptosis factor in MDA-MB-435 cells in response to oligoamine treatment.

Sensitization to oligoamine-induced cell death by TAM67 transfection has also been observed in another human breast cancer cell line, MCF-7. A clone of MCF-7 cells transfected with TAM67 exhibited a similar increase in sensitivity to oligoamines and enhanced $p53/p21^{waf1/cip1}$ pathway activation under oligoamine treatment (data not shown). In addition, activation of the MAPK pathway by a polyamine analogue was recently demonstrated to play a protective role in MALME-3M melanoma cells (23). All these results suggest that activation of the MAPK pathway and its downstream effector, AP-1, may play an important protective role in certain types of tumor cells in response to specific polyamine analogue-induced cell death.

Our studies also investigated the effects of oligoamine on the polyamine metabolic pathway in vector and TAM67 transfected cells (Table 1). ODC is a key regulatory enzyme in polyamine biosynthesis. The significant decrease of ODC activity in SL-11144-treated MDA-MB-435 cells suggests that ODC might be one of the targets for oligoamines. However, it should be noted that the effects of SL-11144 on ODC activity appear to be indirect. When cellular extracts of MDA-MB-435 cells containing ODC enzyme are exposed to concentrations of SL-11144 up to 1000 μM, no change in ODC activity is observed. This is in contrast to results observed with the specific ODC inhibitor, 2-difluoromethylornithine, which produces nearly complete inhibition of ODC activity in cell extracts at concentrations > 100 μM (data not shown). Likely, the cellular effects of SL-11144 resulting in decreased ODC activity are not a result of direct enzyme inhibition but are a result of regulatory mechanisms including the increased production of ODC antizyme as demonstrated by Mitchell *et al.* (52).

In conclusion, we present evidence that treatment with a JNK inhibitor or specific blockade of AP-1 by a dominant-negative mutant c-Jun sensitizes MDA-MB-435 cells to cell death induced by three novel polyamine analogues. The results strongly suggest that the AP-1 proteins play a protective role in this cell line in response to stress and cell death signals. Our work provides new insights into the molecular mechanisms of polyamine analogues in cancer cells. It also suggests that AP-1 may be a useful target for improving the therapeutic efficacy of polyamine analogues in human breast cancer.

Materials and Methods

Compounds, Cell Line, and Culture Conditions

The polyamine analogues SL-11144, SL-11159, and SL-11172 (Fig. 1) were synthesized as reported previously (15, 16). A concentrated stock solution (10 mM in double-distilled water) was diluted with the medium to the desired concentrations for

specific experiments. The selective JNK1, JNK2, and JNK3 inhibitor SP600125 (anthra[1,9-*cd*]pyrazol-6(2*H*)-one) from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA) was prepared as a stock solution of 20 mM in 100% DMSO. The MDA-MB-435 human carcinoma cell line was originally isolated from the pleural effusion of a patient with breast carcinoma (28, 29). It should be noted that one recent report, using microarray analysis, indicated that MDA-MB-435 cells expressed certain markers associated with melanoma (53). However, this report has not been confirmed and is not consistent with the clinical course of the patient from whom the cells were derived. Cells were maintained in improved MEM supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin and incubated at 37°C in a 5% CO_2 atmosphere.

Construction of Expression Plasmid and Stable Transfection of TAM67

The cDNA of mutant c-Jun (TAM67) that lacks amino acids 3–122 of the transactivation domain was kindly provided by Dr. Steve N. Georas (Johns Hopkins) and subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA). Transfections were performed by Lipofect-AMINE Plus reagent (Invitrogen) as recommended by the manufacturer. Stable transfectants were selected by incubating the cells in the medium containing 500 μg/ml geneticin (G418). Cells from individual colonies were examined for TAM67 expression by Western blot analysis with an antibody that recognizes the COOH terminus of c-Jun (sc-44; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Growth Inhibition and Colony Formation Assays

Growth inhibition was assessed by MTT assays as described previously (54). Briefly, 2000–5000 cells were plated in 96-well dishes and treated with the various concentrations of drug regimes for the indicated times. All of the experiments were plated in quadruplicate and were carried out at least thrice. The results of assays were presented as means ± SD. Colony formation assay was performed as published previously (55). Colonies that contained >50 cells were scored. Relative clonogenic efficiency was assessed as numbers of colonies in treated group/numbers of colonies in control group. All experiments were plated in triplicate and were carried out at least thrice. The results were expressed as means ± SD.

Western Blotting

Cells were treated with indicated drug concentrations and times, harvested by trypsinization, and washed with PBS. Cellular protein was isolated using a protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Protein concentrations were determined using Micro Protein Assay Kit (Pierce Chemical Co., Rockford, IL). Equal amounts of protein (50 μg/lane) were fractionated on 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies against c-Jun, c-Fos, caspase-3 (1:2000; Santa Cruz Biotechnology), and PARP (1:2000; Calbiochem, San Diego, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:3000; DAKO Corp., Carpinteria, CA) followed by enhanced chemiluminescent staining using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Actin was used to normalize for protein loading. All the experiments were performed at least twice with similar results.

Analysis of Intracellular Polyamine Pools, SSAT Activity, and ODC Activity

The intracellular polyamine content of treated and untreated cells was determined by precolumn dansylation and reversed phase high-performance liquid chromatography (56). SSAT and ODC activities were measured using cellular extracts as described previously (57, 58). Protein concentrations were determined according to the method of Bradford (59).

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The Distinct Roles for p53/p21$^{WAF1/CIP1}$ and JNK/Jun Activation in the Mediation of Polyamine Analogue Induced Growth Inhibition and Cell Death in Human Breast Cancer Cells

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Abstract:
Intracellular polyamines are absolutely required for cell proliferation and differentiation and many tumors have abnormal requirements for polyamines. Consequently, the polyamine metabolic pathway represents a rational target for antineoplastic intervention. A number of polyamine analogues have been identified to act as potent modulators of cellular polyamine metabolism and possess effective activity against a variety of tumors including breast cancer. In this study we demonstrated that specific analogues exhibited differential inhibitory action against growth of human breast cancer MCF-7 cells. Treatment of MCF-7 cells with oligoamine analogues (SL-11144 and SL-11172) and bis(alkyl)-substituted analogue BENSpm produced a G_1 cell cycle arrest, while another bis(alkyl)-substituted analogue, CHENSpm, induced a G_2/M cell cycle arrest. Each of these compounds significantly up-regulated p53-dependent expression of p21$^{WAF1/CIP1}$ in p53 wild type MCF-7 cells, whereas p21$^{WAF1/CIP1}$ expression was not up-regulated in polyamine analogues treated breast cancer cell lines possessing a mutant p53 gene. These results suggest that polyamine analogue-induced p21$^{WAF1/CIP1}$ expression is p53-dependent. Stable transfection of small interfering RNA (siRNA) targeting p53 blocked the expression of p21$^{WAF1/CIP1}$ induced by the polyamine analogues and significantly inhibited polyamine analogue-induced growth inhibition and apoptosis. Interestingly, the effects of analogue exposure on cyclins and cyclin dependent kinases varied with the specific agent used. These data suggest that regulation of cell cycle arrest by p53/ p21$^{WAF1/CIP1}$ induced by polyamine analogues occurs through agent-specific mechanisms. Furthermore, the use of Jun NH_2-terminal Kinase (JNK) specific inhibitor SP600125 and overexpression of dominant-negative mutant c-Jun (TAM67) sensitized MCF-7 cells to polyamine analogue-induced cell death and promoted the activation of p53 and p21$^{WAF1/CIP1}$. Neither p38 inhibitor nor ERK inhibitor affected polyamine analogue-induced p53 activation and growth inhibition. Taken together, these results suggest that activation of the p53/ p21$^{WAF1/CIP1}$ pathway is important for the induction of polyamine analogue-induced growth inhibition and apoptosis. Whereas JNK/Jun signaling pathway may negatively regulate p53 and play a protective role in MCF-7 cells in response to polyamine analogue treatment.